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(54) Title: USE OF GENETIC MARKERS TO DIAGNOSE FAMILIAL DYSAUTONOMIA

(57) Abstract: Familial Dysautonomia (FD), is an autosomal recessive disorder characterized by developmental arrest in the sensory and autonomic nervous systems and Ashkenazi Jewish ancestry. The familial dysautonomia disease gene (DYS) has previously been mapped to an 11cM segment of chromosome 9q31-33 flanked by D9S53 and D9S105. Using new polymorphic loci, the location of the gene is narrowed to less than 0.5 cM between the markers 43B1GAGT and 157A3. Two markers in this interval, 164D1 and D9S1677, show no recombination with the disease. Haplotype analysis confirmed this candidate region. The identification of these close flanking markers of the familial dysautonomia disease gene allows accurate genetic testing for both familial dysautonomia families and carriers.

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USE OF GENETIC MARKERS TO DIAGNOSE FAMILIAL DYSAUTONOMIA

FIELD OF THE INVENTION

This invention relates to genetic testing, and more specifically, to a method of diagnosing familial dysautonomia in an individual.

BACKGROUND OF THE INVENTION

Familial dysautonomia (FD), or the Riley-Day syndrome, or hereditary sensory neuropathy type III, is a rare inherited neurological disease affecting the development and survival of sensory, sympathetic and some parasympathetic neurons (Axelrod et al., 1974; Axelrod, F.B. 1984; and Axelrod and Pearson 1984). It is the most common and the best known of a group of rare disorders, termed congenital sensory neuropathies, that are characterized by widespread sensory, and variable autonomic dysfunction. Patients with familial dysautonomia are affected from birth with a variety of symptoms including gastrointestinal dysfunction, vomiting crisis, recurrent pneumonias, altered sensitivity to pain and temperature, and cardiovascular instability (Axelrod et al. 1974; Axelrod 1996; Riley et al. 1949). There is progressive neuronal degeneration throughout life and despite recent advances in the management of FD, survival statistics indicate that the probability of reaching 30 years of age is only 50% (Axelrod and Abularrage 1982).

The disorder is inherited as an autosomal recessive with complete penetrance and is largely confined to individuals of Ashkenazi Jewish descent (Brunt, P.W., et al., 1970). In this population, the estimated carrier frequency is 1 in 30 with a disease incidence of 1 in 3600 births (Maayan, C., et al., 1987). The clear-cut pattern of transmission, apparent restriction to one ethnic population and lack of confounding phenocopies suggest that all cases of familial dysautonomia might have descended from a single mutation (Axelrod, F.B. 1984).

The diagnosis of FD is based on the following cardinal criteria: absence of fungiform papillae on the tongue, absence of axon flare after injection of

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intradermal histamine, decreased or absent deep tendon reflexes, absence of overflow emotional tears, and, because of its high prevalence, Ashkenazi Jewish descent (Axelrod 1984; Axelrod and Pearson 1984; Brunt and McKusick 1970). For many years, familial dysautonomia related research concentrated on biochemical, physiological and histological-pathological aspects of the disorder. Although those studies contributed to a better understanding of the nature of the disease, and indicated that a deficiency in a neuronal growth factor pathway might be the cause of familial dysautonomia, they did not result in identification of the familial dysautonomia gene and thus did not contribute to the development of genetic diagnostic test for familial dysautonomia.

Chromosomal localization of the gene causing familial dysautonomia can facilitate genetic counseling and prenatal diagnosis in affected families. Subsequent delineation of closely linked markers which show strong linkage disequilibrium with the disorder and ultimately, identification of the defective gene can allow screening of the entire at-risk population to identify carriers, and potentially reduce the incidence of new cases.

SUMMARY OF THE INVENTION

The present invention relates to a method of diagnosing familial dysautonomia in an individual. More specifically, the invention relates to a method of identifying the inheritance of an allele causing familial dysautonomia by linkage analysis using polymorphic markers of the familial dysautonomia disease gene. The familial dysautonomia disease gene is located between 43B1GAGT and 157A3 on the long arm of human chromosome 9 (q arm). Other markers encompassed by this region include 164D1 and D9S1677. The method provides accurate genetic testing for both familial dysautonomia families and disease carriers.

The invention also relates to nucleic acids and diagnostic kits useful for carrying out genetic testing of familial dysautonomia.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Familial dysautonomia pedigrees showing the recombination events (A, centromeric cross; B, telomeric cross) that define the candidate interval. The shaded bars represent the FD chromosome, unfilled bars are non-FD. The line shows the location of the recombination event.

Figure 2: Extended haplotype analysis of 435 FD chromosomes with 9 markers. The major haplotype is framed at the top. The other haplotypes, believed to be derived from ancestral recombination events, are depicted with the identical FD core markers framed.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to genetic mapping of the familial dysautonomia disease gene. The familial dysautonomia disease gene (*DYS*) has previously been mapped to an 11 centiMorgan (cM) segment of chromosome 9q31-33 flanked by *D9S53* and *D9S105* (Blumenfeld et al., U.S. Patents 5,387,506 and 5,998,133, hereby incorporated in their entirety by reference). One centiMorgan is roughly equivalent to 1,000 kb of DNA. The familial dysautonomia gene is located according to the invention in a gene segment comprising the following sequential polymorphisms: D9S172-D9S261-88B2GA-43B1GAGT-164D1-D9S1677-157A3-D9S310-D9S309-D9S58-D9S160-D9S311-D9S105. The location of the familial dysautonomia gene is narrowed to less than 0.5 cM between the markers 43B1GAGT and 157A3. Two additional markers, 164D1 and D9S1677, located between 43B1GAGT and 157A3, showed no recombination with the disease.

The present invention therefore relates to a method for identifying an individual carrying a gene associated with familial dysautonomia. The method comprises detecting the presence of a polymorphism located between D9S172 and D9S105, preferably between 43B1GAGT and 157A3 inclusive, and most preferably between 164D1 and D9S1677, on human chromosome 9. The presence of such polymorphisms is indicative of the presence of the familial dysautonomia gene in the

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individual.

The polymorphic markers of the invention can be detected by a variety of methods. The preferred detection means uses radioactive nucleotides in PCR amplification of the polymorphism, or randomly labeled probes in hybridization reactions. Other detection methods such as the ligase chain reaction (LCR) can also be used. The polymorphism can be detectably labeled by a radioisotope or by chemical modification enabling direct detection of the polymorphism. Fluorescent or colorimetric means can also be used. Detection of the polymorphism can be indirect, e.g. a radioactive complementary strand of DNA, resulting from incorporation of radioactive nucleotides in a polymerase chain reaction.

The invention also relates to nucleic acids useful for detecting the polymorphic markers of the invention. The nucleic acids, encoding sequences flanking the markers of the invention, can be used as primers for the polymerase chain reaction (PCR). Amplification of DNA with these primers allows for the detection of the polymorphisms such as 88B2GA, 43B1GAGT, 164D1, D9S1677 and 157A3. Such primers may be about 15 to about 40 bases pairs in length, preferably about 17 to about 25 base pairs in length. In a preferred embodiment the primers used are 5'-GCCTGGGTGACAAGAGC-3' or 5'-CTCATTGTATCCTTACATGGTG-3' for the 88B2GA marker; 5'-GATACACCATG-CATTTGC-3' or 5'-GAAA-TAGAACTGTTCCAAG-3' for the 43B1GAGT marker; 5'-CACCAAGTATA-CTCCAGC-3' or 5'-TTAGATAGAAGTTATATTGC-3' for the 164D1 marker; 5'-CTGCTGTAATAGAAGGGAAAGG-3' or 5'-TCAACACCTAAGTCTAATCACC-3' for the 157A3 marker. It will be understood by one of skill in the art that variations in the 88B2GA, 43B1GAGT, 164D1, D9S1677 and 157A3 primers may be made providing they still result in nucleic acid sequences capable of amplifying the corresponding nucleic acid sequence. These primers may be used in the methods described herein for detecting the presence in a subject of the 43B1GAGT, 164D1, D9S1677 and 157A3 polymorphisms.

Using the polymorphic markers of the invention, a genetic test for

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families with familial dysautonomia-affected member is provided for both prenatal diagnosis and carrier test in healthy siblings. Subsequent identification of the defective gene, could also allow screening of the entire at-risk population to identify carriers, and potentially reduce the incidence of new cases of familial dysautonomia.

The method lends itself readily to the formulation of kits which can be utilized in diagnosis. Such a kit would comprise a carrier being compartmentalized to receive in close confinement one or more containers wherein a first container may contain DNA containing coding sequences which may be used to identify a given polymorphism, e.g. an SSR. A second container may contain a different set of sequences coding for a second SSR, and so on. Other containers may contain reagents useful in the detection of the labelled probes, such as enzyme substrates. Still other containers may contain restriction enzymes, buffers, and the like.

The present invention will now be described by way of examples, which are meant to illustrate, but not limit, the scope of the invention.

EXAMPLE 1

Materials and Methods

Familial Dysautonomia Families

Patient samples were obtained from two major sources: the Dysautonomia Diagnostic and Treatment Center at the New York University Medical Center, and the Israeli Center for Familial Dysautonomia at Hadassah University Hospital. All familial dysautonomia (FD) patients included in this study were diagnosed based on the standard criteria previously described (Axelrod 1984; Axelrod and Pearson 1984). Two hundred twelve (212) Ashkenazi FD families were studied, including 41 families with more than one affected member, (siblings, first cousins, and affected uncles/aunts), and two families with consanguinity and a single affected child. Altogether, 271 FD affected individuals (441 distinct FD chromosomes) were studied. Unaffected parents were studied in all 212 FD families, and in 102 of the families siblings, grandparents, and siblings of the parents were also studied (492 non-FD

chromosomes from obligatory carriers). Control chromosomes were obtained from unaffected individuals marrying into the FD families (324 control chromosomes).

Identification of New Markers

Seven new polymorphic markers were generated from cosmids in the FD critical region by hybridization with synthetic di-, tri-, tetra-, and penta-oligonucleotides. Positive cosmids were shotgun subcloned and the positive subclones sequenced. Four of these markers, 157A3, *D9S310*, *D9S309*, and *D9S311*, are (GT)_n repeats, 88B2GA is a (GA)_n repeat, 43B1GAGT is a (GA)_n (GT)_n repeat, and 164D1 is a (AAAAC)_n repeat (Table 1).

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Table 1

Polymorphic Markers in the FD Haplotype

Marker	PCR Primers ^a or Reference	Allele ^b	Allele Frequency ^c	Heterozygosity & CEPH standards
5	88B2GA	GCCTGGGTGACAAGAGC	1(116)	0.38
		CTCATTGTATCCTTACATGGTG	2(118)	0.17
			3(120)	0.01
			4(122)	0.31
			5(124)	0.01
10	43B1GAG T		11(136)	0.12
		GATACACCATGCATTTGC	3(80)	0.01
		GAAAATACAACTGTCCAAG	4(82)	0.03
			5(84)	0.09
			6(86)	0.10
			8(90)	0.61
			9(92)	0.01
			10(94)	0.15
				0.59
				133101 5,8
15	164D1	CACCAGTATACTCCAGC	2(149)	0.01
		TTAGATAGAAGTATATTGC	3(154)	0.15
			4(159)	0.35
			5(164)	0.49
	157A3	CTGCTGTAATAGAAGGGAAAGG	12(140)	0.03
		TCAACACCTAAGTCTAATCACC	13(142)	0.84
			14(144)	0.08
			15(146)	0.02
			16(148)	0.01
			17(150)	0.02
20	D9S310	(Slaugenhaupt et al. 1994)	1	0.02
			2	0.01
			3	0.20
			4	0.07
			5	0.24
			6	0.38
			7	0.08
				0.75
				133101 3,5
				133102 3,3
25	D9S309	(Slaugenhaupt et al. 1994)	1	0.01
			2	0.02
			3	0.02
			4	0.07
			5	0.03
			6	0.08
			7	0.08
			8	0.06
			9	0.37
			10	0.24
30			11	0.01
			15	0.01

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Table 1**Polymorphic Markers in the FD Haplotype**

	Marker	PCR Primers ^a or Reference	Allele ^b	Allele Frequency ^c	Heterozygosity & CEPH standards
5	<i>D9S311</i>	(Slaugenhaupt et al. 1994)	-1	0.01	0.40
			1	0.08	
			3	0.01	133101 6,9
			4	0.01	133102 9,9
			5	0.01	
			6	0.01	
			7	0.10	
			8	0.03	
			9	0.70	
			10	0.01	
			13	0.04	
10	<i>D9S172</i>	(Weissenbach et al. 1992)	1	0.07	0.67
			2	0.48	
			3	0.05	133101 2,4
			4	0.24	133102 2,2
			5	0.10	
			6	0.02	
			7	0.03	
			8	0.01	
15	<i>D9S261</i>	(Gyapay et al. 1994)	0	0.01	0.67
			1	0.07	
			2	0.09	133101 8,8
			3	0.01	133102 2,4
			4	0.17	
			5	0.01	
			6	0.01	
			7	0.05	
			8	0.53	
			10	0.01	
			15	0.04	
20	<i>D9S1677</i>	(Dib et al. 1996)	1	0.01	0.71
			3	0.02	
			4	0.02	133101 9,9
			5	0.02	133102 8,10
			6	0.23	
			7	0.08	
			8	0.14	
			9	0.31	
			10	0.08	
			11	0.05	
			12	0.02	
			13	0.01	
			14	0.01	
25	<i>D9S58</i>	(Kwiatkowski et al. 1992)	1	0.01	0.98
			2	0.01	
			3	0.02	133101 8,10
			4	0.02	133102 7,19
			5	0.03	
			6	0.02	

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Table 1**Polymorphic Markers in the FD Haplotype**

Marker	PCR Primers ^a or Reference	Allele ^b	Allele Frequency ^c	Heterozygosity & CEPH standards
5		7	0.02	
		8	0.06	
		9	0.08	
		10	0.04	
		11	0.05	
		12	0.05	
		13	0.11	
		14	0.11	
		15	0.07	
		16	0.06	
		17	0.01	
		18	0.05	
		19	0.03	
		20	0.10	
		23	0.01	
		24	0.01	
		26	0.03	
15	<i>D9S160</i> (Weissenbach et al. 1992)	-1	0.01	0.72
		0	0.01	
		2	0.03	133101 6,6
		3	0.06	133102 6,7
		4	0.10	
		5	0.06	
		6	0.34	
		7	0.38	
20	<i>D9S105</i> (Weber 1991)	1	0.02	0.83
		2	0.07	
		3	0.14	133101 8,8
		4	0.14	133102 4,8
		5	0.08	
		6	0.06	
		7	0.11	
		8	0.31	
25		9	0.05	
		10	0.01	
		11	0.01	

^aNew polymorphisms, PCR primers are listed 5' - 3'^bAlleles sizes in base pairs for new markers^cAllele frequencies are based on 497 non-FD Ashkenazi Jewish chromosomes

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DNA Analysis

Genomic DNA was either prepared from lymphoblast cell lines (Anderson and Gusella 1984), using the SDS-proteinase K method, followed by phenol extraction, or directly from blood, using the Chelex-100 method (Walsh et al. 1991). PCR analysis was carried out on genomic DNA using the published oligonucleotide primer pairs and annealing temperatures (Dib et al. 1996; Gyapay et al. 1994; Kwiatkowski et al. 1992; Weissenbach et al. 1992, The Genome Database) or according to Table 1. Typing of SSR polymorphisms was performed as described in (Blumenfeld et al. 1993b).

RESULTS

Order of markers in the *DYS* region

Previous studies have localized the FD gene close to *D9S58*, in an 11 cM region between *D9S53* and *D9S105*. In the present study, thirteen SSR polymorphisms from the *DYS* region were used, including both *D9S58* and *D9S105*. On the proximal side, the closer marker *D9S172* (6 cM from *D9S58*) was substituted for the more distant marker *D9S53* (8 cM from *D9S58*). The order of the ten additional markers (Table 1) with respect to the aforementioned three anchoring loci from centromere to telomere is:
centromere-*D9S172*-*D9S261*-88B2GA-43B1GAGT-164D1-*D9S1677*-157A3-*D9S310*-*D9S309*-*D9S58*-*D9S160*-*D9S311*-*D9S105*-telomere.

This map order was determined sequentially from recombination events in reference pedigrees (Povey et al. 1997) and recombination events in our FD families. No crossovers were observed between 164D1 and *D9S1677*, but their relative order was established by isolation of a BAC clone containing *D9S1677* and 157A3 but not 164D1.

Fine localization of the FD Gene

To refine the minimum FD candidate region, one hundred and two (102) FD families (41 with multiple affecteds) were analyzed. On the proximal side,

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the recombination event depicted in Figure 1A sets the closest centromeric flanking marker as 43B1GAGT. No additional crossovers were detected by 88B2GA or *D9S261*, although the more distant *D9S172* (~6 cM away) detected 14 recombinations with *DYS*. On the distal side, the closest flanking marker is 157A3 based on the crossover shown in Figure 1B. One additional crossover was found in each of the subsequent intervals: 157A3-*D9S309*, *D9S309*-*D9S310*, and *D9S309*-*D9S58*. No recombinants were observed between *DYS* and 164D1-*D9S1677*. Thus, the FD candidate region has been reduced to the interval 43B1GAGT-164D1-*D9S1677*-157A3, which we estimate from the analysis to span less than 0.5 cM.

A Major FD Haplotype

Haplotype analysis of FD was carried out in an attempt to further refine the candidate region and to estimate the number of independent mutations represented in the FD population. A major founder haplotype was observed for 435 of the 441 (98.6%) FD chromosomes examined, with a core of alleles '8-4-12' at 43B1GAGT-164D1-*D9S1677* (Table 2) and a consensus set of alleles for markers on either side that decays due to historical recombination events. The major founder haplotype in FD is recognizable across the interval *D9S261* to *D9S105*, approximately 3 cM. The chromosomes supporting historical recombinations across the *D9S261* to *D9S58* interval are depicted in Figure 2. No events were detected to narrow the candidate region, although one ancestral recombination event with 157A3 was observed which confirms it as the closest telomeric flanking marker (Figure 2). The next distal flanking markers, *D9S310* and *D9S309*, yielded evidence for 4 and 6 additional ancestral recombinations, respectively. On the centromeric side, 3 apparent ancestral recombinations were observed with 88B2GA, and 6 additional events were seen with *D9S261*.

D9S1677 forms part of the conserved haplotype, but displays some allelic variation due to 'slippage' events that create new alleles (Table 3). On most 'major haplotype' FD chromosomes, *D9S1677* is represented by a '12' allele (83.5%), but on the remainder it is represented by '10' (0.5%), '11' (3%), '13' (3%), or '14'

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(10%) even though adjacent markers remain unchanged. By contrast, the extreme *D9S1677* alleles '12', '13' and '14' are present on only 2.4%, 0.6% and 0.6%, respectively, of non-FD chromosomes. The instability of *D9S1677* is further supported by our observation of two allele changes from '12' to '13' during parent-child transmissions in our FD families.

Other FD Haplotypes

Six of 441 FD chromosomes revealed three different haplotypes across the candidate region (Table 2). All three of these other haplotypes were observed in compound heterozygotes with the major haplotype. Minor haplotypes 1, 2, and 3 were observed in two, three, and one unrelated families, respectively. The third rare haplotype was inherited from a woman who claimed not to be of Jewish extraction; she was of Irish-German/Sicilian origin. Other than the unusual family history, this child exhibited all of the diagnostic criteria for FD and had classical symptoms.

Haplotypes Associated with FD

***10,11,12,13,14 (see Table 3)**

^aSee Figure 2 for details

Allele variation observed at D9S1677

<i>D9S261</i>	8B2GA	43B1GAGT	164D1	<i>D9S1677</i>	157A3	<i>D9S310</i>	<i>D9S309</i>
8	4	8	4	10	13	5	10
8	4	8	4	11	13	5	10
x	x	8	4	12	13	x	x
x	4	8	4	13	13	5	10
						6	9
						6	9
x	4	8	4	14	13	5	10
						6	9
						6	9
						6	6

See Figure 2 for details of x alleles

DISCUSSION

In an extensive study of FD families undertaken to refine the location of the FD gene in 9q31, eleven new polymorphic markers were used. Within the 11 cM candidate region previously reported (Blumenfeld et al. 1993b), recombination events in FD families that define a FD gene candidate region of less than 0.5 cM, between the new markers 43B1GAGT and 157A3 were observed.

One major haplotype for the FD region was detected on more than 98% of FD chromosomes. Indeed, all of the FD patients studied have at least one copy of the major haplotype. This dramatic linkage disequilibrium indicates that one major founder mutation is responsible for virtually all FD cases in the Ashkenazim. In several other recessive hereditary diseases a major founder mutation has been observed in Ashkenazi Jews, but in none of them is a single founder mutation as preponderant as the haplotype found in FD. For example the major mutations found in Tay Sachs disease, Gaucher disease, and cystic fibrosis are observed on 78%, 76%, and 48% of disease chromosomes, respectively (Triggs-Raine et al. 1990; Beutler et al. 1993; Abeliovich et al. 1992).

Of our 271 FD patients, only 9 individuals from six families were compound heterozygotes with one atypical haplotype. These three rare haplotypes may reflect independent FD mutations. In particular, the inheritance of haplotype 3 from a non-Ashkenazi parent suggests that at least one of the rare FD haplotypes may have been introduced from a non-Ashkenazi population. However, the possibility that haplotypes 1 and 2 represent mutations that have occurred more recently in the Ashkenazim cannot be ruled out. In addition, one of the minor haplotypes observed in two affected individuals (haplotype #1, Table 2), has the same alleles as the major haplotype for the centromeric markers *D9S172* to 43B1GAGT. Compound heterozygotes for this haplotype appear to express a classic FD phenotype. Therefore, this haplotype could conceivably result from a historical recombination event with the major haplotype between 43B1GAGT and 164D1, rather than representing an independent mutation.

If haplotype 1 is a derivative of the major FD haplotype, this would position *DYS* between 164D1 and the flanking marker 43B1GAGT. Haplotypes on non-FD Ashkenazi chromosomes can also be interpreted as providing tentative support for a location of the disease gene proximal to *D9S1677*. None of the 497 non-FD chromosomes tested has

a haplotype that matches the consensus FD haplotype. However, careful examination of those non-FD haplotypes with alleles 11-14 at *D9S1677* revealed 4 chromosomes that have the haplotype (11,12)-13-6-9-13-7-9-X for the markers *D9S1677-157A3-D9S310-D9S309-D9S58-D9S160-D9S311-D9S105*, matching the distal portion of the haplotype observed on 40 out of 435 FD chromosomes (9%, Figure 2). Centromeric to *D9S1677*, all four non-FD chromosomes have the haplotype 11-5-5 instead of 4-8-4 for the markers 88B2GA-43B1GAGT-164D1. It is intriguing to speculate that these non-FD chromosomes may reflect a historical recombination event telomeric to 164D1 which would place the *DYS* gene proximal to *D9S1677*. Although we do not feel that our current data provide strong enough evidence for definitively refining the localization of *DYS* within the 43B1GAGT-164D1-*D9S1677-157A3* interval, these interpretations of rare haplotype 1 and of the selected non-FD chromosomes favor the centromeric portion of the candidate region.

The FD candidate region now extends from 43B1GAGT to 157A3, defined on each side by an actual recombination event observed in a parent-child transmission in one of our FD families. In other studies, haplotype analysis has assisted in pinpointing the location of a disease gene within a candidate interval previously defined by actual recombinants. For example, in Ashkenazi Jewish dystonia, haplotype analysis reduced the interval containing the *DYT1* gene from approximately 1.8 Mb to 150 kb (Ozelius et al. 1997). Similarly, in Huntington's disease, haplotype studies revealing ancestral crossovers progressively narrowed a 2 Mb candidate region to ~200 kb (Gusella and MacDonald 1993). We observe significant linkage disequilibrium on FD chromosomes across a region of about 3 cM from *D9S261* to *D9S105*. Despite the fact that we genotyped a very large number of FD chromosomes, the candidate region could not be narrowed further using ancestral recombination events. Only one additional historical recombination event was observed with 157A3, and none was seen with 43B1GAGT (Figure 2). Thus, the extent of linkage disequilibrium on FD chromosomes and the comparison of historical and actual recombination events in FD suggests that the major FD mutation probably occurred relatively recently in the Ashkenazi population, certainly within a few hundred years. The high incidence of FD in the Ashkenazim suggests that the mutation was likely present during a period of rapid population expansion from a small number of founders (Risch et al. 1995).

The incidence of FD is 1 in 3700 live births among Ashkenazi Jews, and the calculated carrier frequency is 1 in 32 individuals (Maayan et al. 1987). 324 control chromosomes from spouses of FD carriers were genotyped and the major FD haplotype was observed on 1.54% (expected 1.56%). The fact that none of the FD associated haplotypes was observed in non-FD chromosomes, combined with the ability to identify the major FD haplotype in the general Ashkenazi Jewish population, indicates that accurate and sensitive genetic testing can be provided for FD families and spouses (Blumenfeld et al. 1995; Eng et al. 1995; Oddoux et al. 1995).

The definition of a precise candidate region for *DYS* has set the stage for the identification of the the FD defect through location cloning. FD belongs to a family of hereditary sensory neuropathies whose accurate diagnosis challenges clinicians. The observation of at least one non-Jewish FD chromosome in our data indicates that other non-Jewish patients might have escaped diagnosis as FD. Cloning of the *DYS* gene based on its chromosomal location will provide the means for direct comparison of both 'atypical' cases as well as other sensory neuropathies to FD, allowing a classification based on the primary genetic cause rather than subtle symptomatic differences.

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References

- Abeliovich D, Lavon IP, Lerer I, Cohen T, Springer C, Avital A, Cutting G (1992) Screening for five mutations detects 97% of cystic fibrosis (CF) chromosomes and predicts a carrier frequency of 1:29 in the Jewish Ashkenasi population. *Am J Hum Genet* 51:951-956
- Anderson MA, Gusella JF (1984) Use of cyclosporin A in establishing Epstein-Barr virus-transformed human lymphoblastoid cell lines. *In Vitro* 20:856-8
- Axelrod FB, Nachtigal R, Dancis J (1974) Familial dysautonomia: diagnosis, pathogenesis and management. *Adv Pediatr* 21:75-96
- 5 Axelrod FB (1984) Familial dysautonomia and other congenital and sensory autonomic neuropathies. In: Blake IB (ed) *Cell and Molecular Biology of Neuronal Development*. Plenum Press, New York, pp 331-340
- Axelrod FB, Pearson J (1984) Congenital sensory neuropathies. Diagnostic distinction from familial dysautonomia. *Am J Dis Child* 138:947-54
- 10 Axelrod FB, Abularrage JJ (1982) Familial Dysautonomia. A prospective study of survival. *J Pediatr* 101:234-236
- Axelrod FB (1995) Familial dysautonomia. In: Robertson D, Biaggioni I (eds) *Disorders of the Autonomic Nervous System*. Vol. 5. Harwood Academic Publishers, Luxembourg, pp 217-231
- 15 Axelrod FB (1996) Familial dysautonomia. In: Robertson D, Low PA, Polinsky RJ (eds) *Primer on the Autonomic Nervous System*. Academic Press, San Diego, pp 242-249
- Beutler E, Nguyen NJ, Henneberger MW, Smolec JM, McPherson RA, West C, Gelbart T (1993) Gaucher Disease: gene frequencies in the Ashkenazi Jewish population. *Am J Hum Genet* 52:85-88
- 20 Blumenfeld A, Slaugenhaupt SA, Axelrod FB, Lucente DE, Maayan C, Liebert CB, Ozelius LJ, et al (1993a) Localization of the gene for familial dysautonomia on chromosome 9 and definition of DNA markers for genetic diagnosis. *Nat Genet* 4:160-4
- Blumenfeld A, Axelrod FB, Trofatter JA, Maayan C, Lucente DE, Slaugenhaupt SA, Liebert CB, et al (1993b) Exclusion of familial dysautonomia from more than 60% of the genome. *J Med Genet* 30:47-52
- 25 Blumenfeld A, Axelrod FB, Tamper V, Maayan C (1995) [Localization of the familial dysautonomia gene to chromosome 9q31-33 and the development of a genetic test for the disease]. *Isr J Med Ass* 128:97-100
- Breakefield XO, Orloff G, Castiglione C, Coussens L, Axelrod FB, Ullrich A (1984) Structural gene for beta-nerve growth factor not defective in familial dysautonomia. *Proc Natl Acad Sci U S A* 81:4213-6
- 30 Breakefield XO, Ozelius L, Bothwell MA, Chao MV, Axelrod F, Kramer PL, Kidd KK, et al (1986) DNA polymorphisms for the nerve growth factor receptor gene exclude its role in familial dysautonomia. *Mol Biol Med* 3:483-94

- Brunt PW, McKusick VA (1970) Familial dysautonomia. A report of genetic and clinical studies, with a review of the literature. *Medicine (Baltimore)* 49:343-74
- Dib C, Faure S, Fizames C, Samson D, Drouot N, Vignal A, Millasseau P, et al (1996) A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature* 380:152-4
- Eng CM, Slaugenhaupt SA, Blumenfeld A, Axelrod FB, Gusella JF, Desnick RJ (1995) Prenatal diagnosis of familial dysautonomia by analysis of linked CA-repeat polymorphisms on chromosome 9q31-q33. *Am J Med Genet* 59:349-55
- Gusella JF, MacDonald ME (1993) Hunting for Huntington's disease. *Mol Genet Med* 5 3:139-58
- Gyapay G, Morissette J, Vignal A, Dib C, Fizames C, Millasseau P, Marc S, et al (1994) The 1993-94 Genethon human genetic linkage map. *Nat Genet* 7:246-339
- Harris DJ, Yang BI, Wolf B, Snodgrass PJ (1980) Dysautonomia in an infant with secondary hyperammonemia due to propionyl coenzyme A carboxylase deficiency. *Pediatrics* 10 65(1):107-110
- Klebanoff MA and Neff JM (1980) Familial dysautonomia associated with recurrent osteomyelitis in a non-Jewish girl. *J Pediatr* 96(1):75-77
- Kwiatkowski DJ, Henske EP, Weimer K, Ozelius L, Gusella JF, Haines J (1992) Construction of a GT polymorphism map of human 9q. *Genomics* 12:229-40
- Levine SL, Manniello RF, Farrell PM (1977) Familial dysautonomia: unusual presentation in an infant on non-Jewish ancestry. *J Pediatr* 90(1):79-81 15
- Maayan C, Kaplan E, Shachar S, Peleg O, Godfrey S (1987) Incidence of familial dysautonomia in Israel 1977-1981. *Clin Genet* 32:106-8
- Metha K (1978) Familial dysautonomia in a Hindu boy. *Am J Dis Child* 32:719
- Orbeck H and Oftedal G (1977) Familial dysautonomia in a non-Jewish child. *Acta Paediatr Scand* 66(6):777-781 20
- Ozelius LJ, Hewett J, Kramer P, Bressman SB, Shalish C, de Leon D, Rutter M, et al (1997) Fine localization of the torsion dystonia gene (DYT1) on human chromosome 9q34: YAC map and linkage disequilibrium. *Genome Res* 7:483-94
- Oddoux C, Reich E, Axelrod F, Blumenfeld A, Maayan C, Slaugenhaupt S, Gusella J, et al (1995) Prenatal diagnostic testing for familial dysautonomia using linked genetic markers. 25 *Prenat Diagn* 15:817-26
- Pearson J, Pytel BA (1978a) Quantitative studies of sympathetic ganglia and spinal cord medio-lateral gray columns in familial dysautonomia. *J Neurol Sci* 39:47-59
- Pearson J, Pytel B (1978b) Quantitative studies of ciliary and sphenopalatine ganglia in familial dysautonomia. *J Neurol Sci* 39:123-30
- Pearson J, Pytel BA, Grover-Johnson N, Axelrod F, Dancis J (1978) Quantitative studies of dorsal root ganglia and neuropathologic observations on spinal cords in familial dysautonomia. *J Neurol Sci* 35:77-92 30

- Povey S, Attwood J, Chadwick B, Frezal J, Haines JL, Knowles M, Kwiatkowski DJ, et al (1997) Report on the Fifth International Workshop on Chromosome 9 held at Eynsham, Oxfordshire, UK, September 4-6, 1996. *Ann Hum Genet* 61:183-206
- Riley CM, Day RL, Greely D, Langford WS (1949) Central autonomic dysfunction with defective lacrimation. *Pediatrics* 3:468-477
- Risch N, de Leon D, Ozelius L, Kramer P, Almasy L, Singer B, Fahn S, et al (1995) Genetic analysis of idiopathic torsion dystonia in Ashkenazi Jews and their recent descent from a small founder population. *Nat Genet* 9:152-9
- Schwartz JP, Breakefield XO (1980) Altered nerve growth factor in fibroblasts from patients with familial dysautonomia. *Proc Natl Acad Sci U S A* 77:1154-8
- Slaugenhaupt SA, Blumenfeld A, Liebert CB, Mull J, MacCormack K, LeBel A, O'Leary K, et al. (1994) Physical map surrounding the familial dysautonomia gene. In: Pericak-Vance MA, Bale AE, Haines JL, Kwiatkowski DJ, Pilz A, Slaugenhaupt SA, White JA, et al. Report on the Fourth International Workshop on Chromosome 9. *Ann Hum Genet* 59:347-365
- 10 Slaugenhaupt SA, Blumenfeld A, Liebert CB, Mull J, Lucente DE, Monahan M, Breakefield XO, et al. (1995) The human gene for neurotrophic tyrosine kinase receptor type 2 is located on chromosome 9 but is not the familial dysautonomia gene. *Genomics* 25:730-732.
- Suzuki T, Higa S, Hayashi A, Nakagawa T, Fujii K (1976) A case of new dysautonomia-like disorder found in Japan. I. Clinical and metabolic studies. *Eur Neurol* 14(2):146-160.
- 15 Triggs-Raine BL, Feigenbaum ASJ, Natowicz M, Skomorowski MA, Schuster SM, Clarke JTR, Mahuran DJ, et al. (1990) Screening for carriers of Tay-Sachs disease among Ashkenazi Jews: a comparison of DNA-based and enzyme-based tests. *New Eng J Med* 323:6-12
- Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* 10:506-13
- 20 Weissenbach J, Gyapay G, Dib C, Vignal A, Morissette J, Millasseau P, Vaysseix G, et al (1992) A second-generation linkage map of the human genome. *Nature* 359:794-801
- Wrathall JR (1986) Reduced neuronotrophic activity of fibroblasts from individuals with dysautonomia in cultures of newborn mouse sensory ganglion cells. *Brain Res* 364:23-9

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We claim:

1. A method for identifying an individual carrying a gene associated with familial dysautonomia, said method comprising:
 - detecting the presence of a polymorphism located between D9S172 and D9S105 inclusive on human chromosome 9, linked to the gene associated with familial dysautonomia, wherein the presence of the polymorphism is indicative of the presence of the familial dysautonomia gene.
2. The method according to claim 1, wherein the polymorphism is selected from the group consisting of D9S172, D9S261, 88B2GA, 43B1GAGT, 164D1, D9S1677, 157A3, D9S310, D9S309, D9S58, D9S160, D9S311 and D9S105.
3. The method according to claim 2, wherein the polymorphism is selected from the group consisting of 43B1GAGT, 164D1, D9S1677 and 157A3.
4. The method according to claim 3, wherein the polymorphism is selected from the group consisting of 164D1 and D9S1677.
5. An isolated nucleic acid sequence comprising the familial dysautonomia gene, said nucleic acid sequence consisting essentially of the sequence located on the long arm of chromosome 9 between 43B1GAGT and 157A3.
6. The isolated nucleic acid sequence according to claim 5, wherein the sequence consists essentially of the sequence located on the long arm of chromosome 9 between 164D1 and D9S1677.
7. A nucleic acid primer for detecting the 43B1GAGT marker, said primer having a nucleic acid sequence of 5'-GATACACCATGCATTTGC-3' or 5'-GAAATAGAACTGTTCCAAG-3'.
8. A nucleic acid primer for detecting the 164D1 marker, said primer having a nucleic acid sequence of 5'-CACCAGTATACTCCAGC-3' or 5'-TTAGATAGAAGTTATATTGC-3'.

9. A nucleic acid primer for detecting the 157A3 marker, said primer having a nucleic acid sequence of 5'-CTGCTGTAATAGAAGGGAAAGG-3' or 5'-TCAACACCTAAGTCTAATCACC-3'.
10. A nucleic acid primer for detecting the 88B2GA marker, said primer having a nucleic acid sequence of 5'-GCCTGGGTGACAAGAGC-3' or 5'-CTCATTGTATCCTTACATGGTG-3'.
- 5 11. A kit for diagnosing a carrier of the familial dysautonomia gene, said kit comprising at least one nucleic acid probe having a sequence that can identify a polymorphism between D9S172 and D9S105 inclusive linked to the gene associated with familial dysautonomia.
- 10 12. The kit according to claim 11, wherein the nucleic acid probe has a sequence that can identify a polymorphism between 43B1GAGT and 157A3 inclusive linked to the gene associated with familial dysautonomia.
- 15 13. The kit according to claim 12, wherein the nucleic acid probe has a sequence that can identify a polymorphism between 164D1 and D9S1677 inclusive linked to the gene associated with familial dysautonomia.

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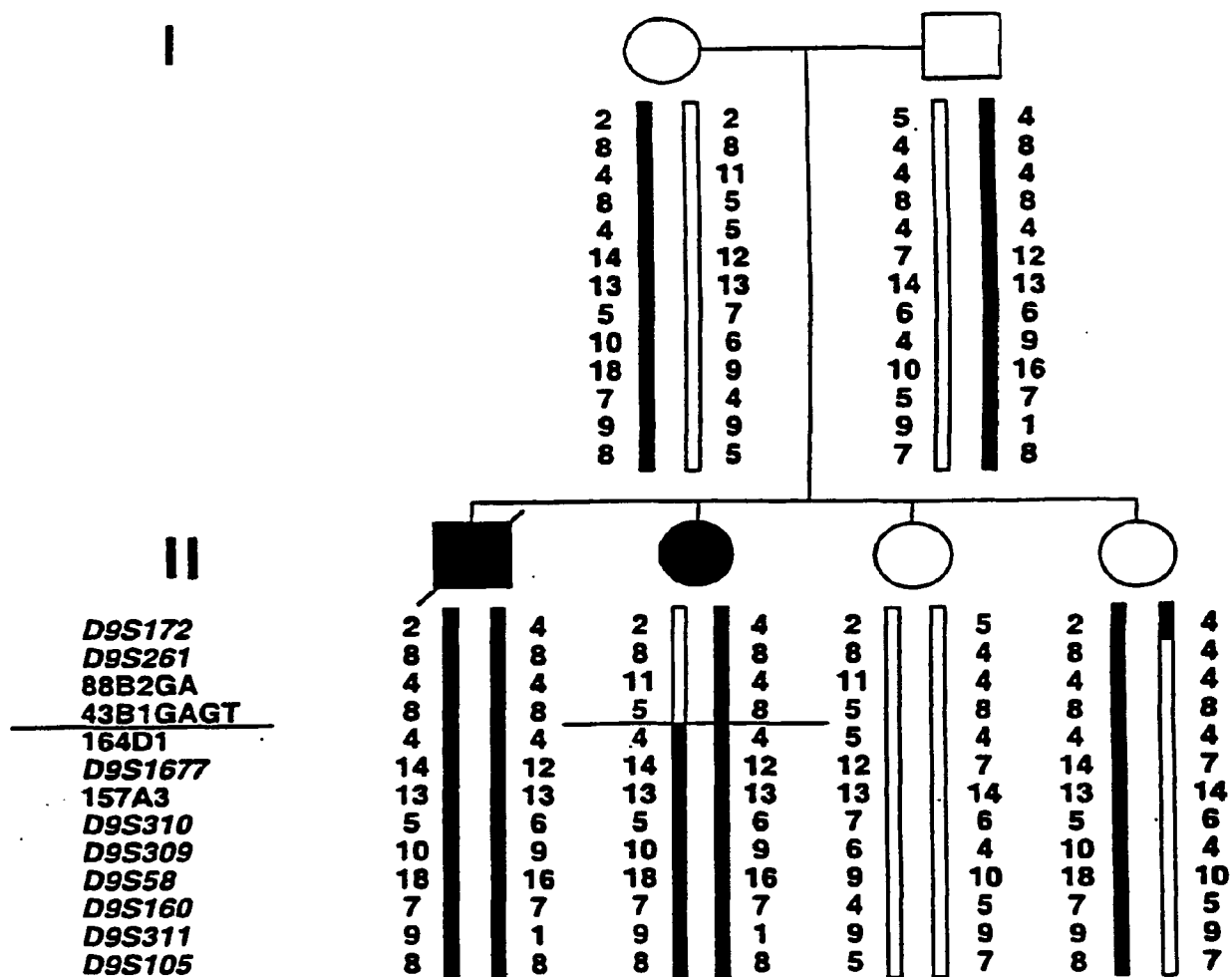


FIG. 1A

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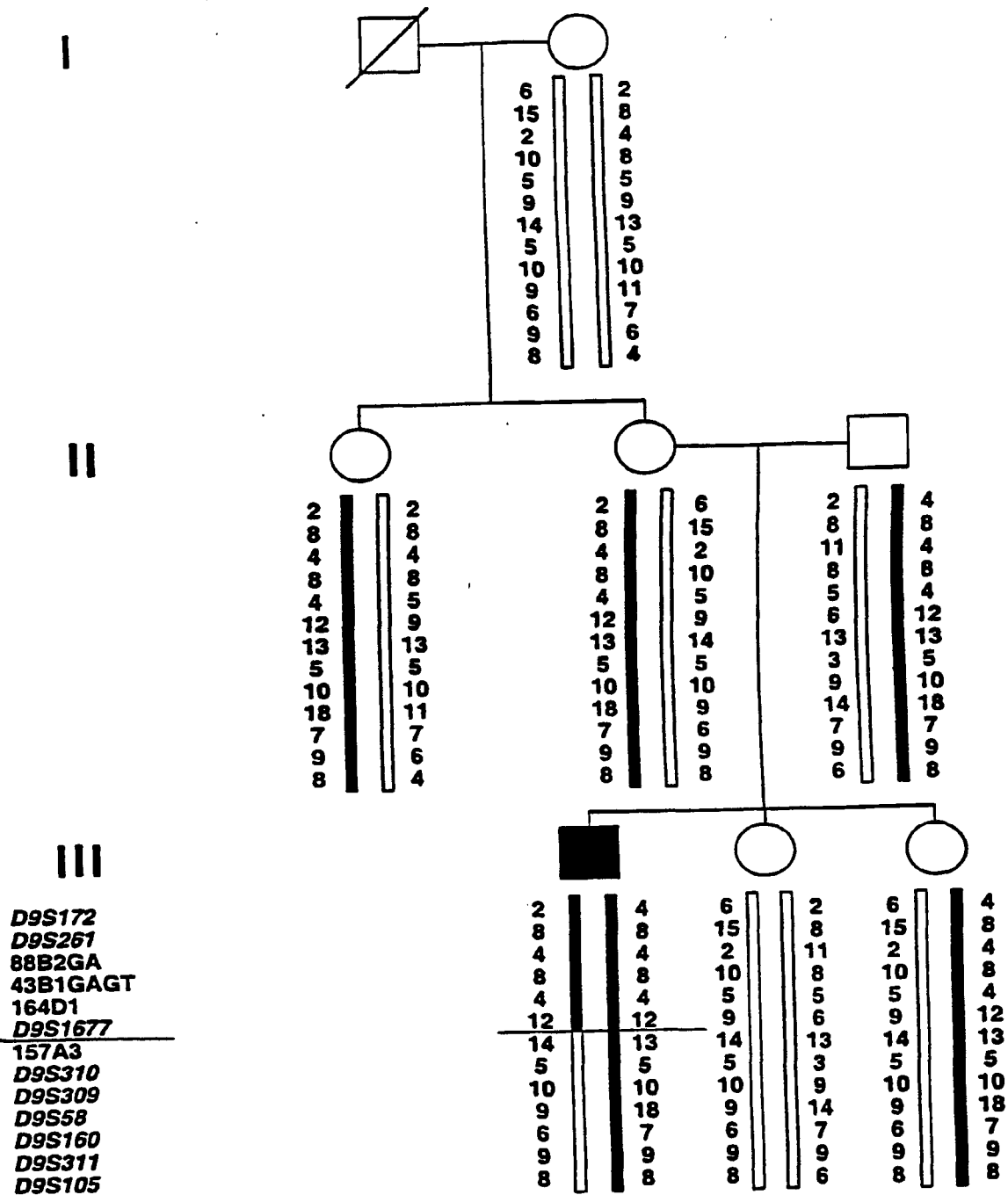


FIG. 1B

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D9S281	88B2GA	43B1GAGT	164D1	D9S1677	157A3	D9S310	D9S309	D9S38	# chromosomes
8	4	8	4	12*	13	5	10	18	307
8	4	8	4	12*	13	6	9	13	40
8	4	8	4	12*	13	6	9	16	15
2	1	8	4	12	13	5	10	18	1
8	1	8	4	12	13	5	10	18	7
8	1	8	4	12	13	6	9	8	1
8	2	8	4	12	13	5	10	18	2
8	11	8	4	12	13	6	9	16	1
1	4	8	4	12	13	5	10	18	2
4	4	8	4	12*	13	5	10	18	8
4	4	8	4	13	13	6	9	13	1
4	4	8	4	14*	13	6	9	16	2
4	4	8	4	12	13	6	10	14	1
7	4	8	4	12	13	5	10	18	8
7	4	8	4	12	13	5	9	19	1
7	4	8	4	13	13	6	9	13	1
7	4	8	4	14	13	6	9	16	1
13	4	8	4	12	13	5	10	18	2
15	4	8	4	12	13	5	10	18	3
15	4	8	4	14	13	6	6	7	1
8	4	8	4	12	14	6	6	8	1
8	4	8	4	12	13	2	8	4	1
8	4	8	4	12	13	2	8	5	3
8	4	8	4	12	13	3	10	24	1
8	4	8	4	12	13	5	1	18	1
8	4	8	4	12	13	5	9	8	1
8	4	8	4	12	13	5	9	9	1
8	4	8	4	12	13	5	9	12	1
8	4	8	4	12	13	5	9	19	2
8	4	8	4	12	13	5	10	3	1
8	4	8	4	12	13	5	10	24	1
8	4	8	4	12	13	6	1	20	1
8	4	8	4	12	13	6	2	20	1
8	4	8	4	12	13	6	2	22	1
8	4	8	4	14	13	6	6	7	1
8	4	8	4	12	13	6	9	9	3
8	4	8	4	12	13	6	9	15	3
8	4	8	4	12	13	6	9	26	1
8	4	8	4	12	13	6	10	8	2
8	4	8	4	12	13	6	10	14	1
8	4	8	4	12	13	7	6	5	2

*alleles = 10,11,12,13,14 (see Table 3)

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/06851

A. CLASSIFICATION OF SUBJECT MATTER														
IPC(7) : C12Q 1/68; C07H 21/04														
US CL : 435/6; 536/23.1, 24.3, 24.3														
According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED														
Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/6; 536/23.1, 24.3, 24.3														
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched														
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Continuation Sheet														
C. DOCUMENTS CONSIDERED TO BE RELEVANT														
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.												
X	BLUMENFELD et al. "Precise Genetic Mapping and Haplotype Analysis of the Familial Dysautonomia Gene on Human Chromosome 9q31". American J. of Human Genetics, April 1999, Vol 64, No. 4, pages 1110-1118.	1-6, 11-13												
Y	US 5,998,133 A (BLUMENFELD et al) 07 December 1999 (07.12.1999), see entire document.	1-6, 11-13												
Y	SLAUGENHAUPT et al. "Refinement of the candidate region and isolation of candidate genes for Familial Dysautonomia on human chromosome 9q31". Am. J. of Human Genetics, Article 287, October 1996, Vol 59, No. 4, pg A55.	1-6, 11-13												
Y	ODDOUX et al "Prenatal Diagnostic Testing for Familial Dysautonomia using linked genetic markers". Prenatal Diagnosis, September 1995, Vol 15, No. 9, pages 817-826.	1-6, 11-13												
Y	ENG et al. "Prenatal Diagnosis of Familial Dysautonomia by analysis of linked CA-Repeat polymorphisms on Chromosome 9q31-q33". Am. J. of Medical Genetics, November 1995, Vol. 59, No. 3, pages 349-355.	1-6, 11-13												
Y	BLUMENFELD et al. "Localization of the gene for familial dysautonomia on chromosome 9 and definition of DNA markers for genetic diagnosis". Nature Genetics, June 1993, Vol. 4, pages 160-164.	1-13												
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.														
<table border="0"> <tr> <td colspan="2">* Special categories of cited documents:</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"E" earlier application or patent published on or after the international filing date</td> <td>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td>"&" document member of the same patent family</td> </tr> </table>			* Special categories of cited documents:		"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"A" document defining the general state of the art which is not considered to be of particular relevance	"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"O" document referring to an oral disclosure, use, exhibition or other means	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"P" document published prior to the international filing date but later than the priority date claimed		"&" document member of the same patent family
* Special categories of cited documents:		"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention												
"A" document defining the general state of the art which is not considered to be of particular relevance	"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone												
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"O" document referring to an oral disclosure, use, exhibition or other means	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art												
"P" document published prior to the international filing date but later than the priority date claimed		"&" document member of the same patent family												
Date of the actual completion of the international search 15 May 2000 (15.05.2000)		Date of mailing of the international search report 03 AUG 2000												
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230		Authorized officer Jeanine Enwold Telephone No. (703) 308-0196												

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/06851

Continuation of B. FIELDS SEARCHED Item 3: Medline, Biosis, Caplus, Embase, Scisearch
search terms: familial dysautonomia, riley-day, hereditary sensory, fd, gene, marker, localization, 43B1GAGT,
157A3, 164D1, D9S1677

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/06851

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claim Nos.: 7-10
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
No computer readable form of the sequence listing was furnished.
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☐

No protest accompanied the payment of additional search fees.